Final Report
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Immunological Influences on the Vestibular System
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The goals of this project were to examine the influence of immune signaling molecules on the survival and replacement of sensory hair cells in the vestibular organs. We have made considerable progress toward that goal, particularly in the characterization of mechanisms that underlie hair cell death.

(I)Immunological Influences on Vestibular Regeneration

Our previous studies had shown that increased numbers of macrophages are recruited to sites of hair cell lesions in organ cultures of the avian cochlea and utricle. Although those observations suggested a role for macrophages and their cytokine secretory products in the regenerative process, the precise role of immune cells in regeneration remained unclear. It is reasonable to assume that if the secretory products of macrophages help to stimulate the regenerative proliferation of supporting cells, then pharmacological inhibition of cytokine production by macrophages should lead to a corresponding decrease in proliferation. Macrophage secretion of immune cytokines can be inhibited by application of glucocorticoid immunosuppressents such as dexamethasone, and a series of experiments examined the effects of dexamethasone on regenerative proliferation in the avian utricle. Prior studies of vestibular regeneration (conducted *in vitro*) suggested that dexamethasone treatment can reduce the levels of supporting cell proliferation. We extended these findings to demonstrate that systemic injections of dexamethasone can also reduce regenerative proliferation *in vivo*. One set of experiments examined the effects of immunosuppression on normal ongoing proliferation of vestibular supporting cells. Chicks (n=10) were given subcutaneous injections of dexamethasone (10 mg/kg) for four days and were then euthanized two hours after an injection of BrdU (100 mg/kg, i.p.). Utricles from the dexamethasone-treated animals contained 139±26 BrdU-labeled supporting cells, compared with 185±17 labeled cells in the controls. Additional experiments examined the effects of dexamethasone treatment on regenerative proliferation following application of aminoglycosides. Chicks received a single injection of gentamicin (250 mg/kg) and then received injections of dexamethasone (10 mg/kg) for four days. Proliferating cells were labeled as described above. Utricles from dexamethasone-treated chicks contained 134±29 BrdU-labeled supporting cells, compared with 226±45 labeled cells in the controls. These results suggest that suppression of macrophage activation can reduce the extent of hair cell regeneration in the avian vestibular organs, and have appeared in published form (Warchol et al., 2001)

Effects of Identified Immune Cytokines on Supporting Cell Proliferation

Activated macrophages secrete a number of cytokines that could potentially influence the proliferation of inner ear supporting cells. In order to identify cytokines that may be involved in regeneration, cultures of isolated sensory epithelia were treated for 48 hours with IL-1β, LIF, TGF-α, TGF-β1, or TNF-α. Cytokines were applied at concentrations of 10 ng/ml and proliferating cells were labeled by adding BrdU to the culture medium during the final four hours *in vitro*. Quantification of BrdU-labeled cells was carried out in confluent regions of 20-40 cells/10,000 μm^2. The proliferation index in untreated (control) cultures ranged from 0.11-0.17. Treatment with TGF-α and TNF-α increased the level of supporting cell proliferation, by 155±9% and 142±17%, respectively. In contrast, treatment with TGF-β1 decreased supporting cell proliferation to 69±6% of control values. Both IL-1β and LIF had no measurable effect on supporting cell proliferation. These results suggest a mechanism by which macrophages and other leukocytes could influence sensory regeneration in the inner ear.

(II) Mechanisms of Hair Cell Death

Studies of other tissue types have suggested that resident macrophages recognize and remove cells that are undergoing programmed cell death (apoptosis). In addition, other

studies have suggested that resident macrophages might also *cause* cells to enter an apoptotic pathway. It became evident that, in order to fully understand the role of macrophages in the inner ear, we must first understand how hair cells die. One of the morphological hallmarks of programmed cell death is chromatin condensation and DNA fragmentation. To examine changes in the nuclear morphology in hair cells after aminoglycoside treatment, utricles were cultured for 24 hours in the presence or absence of 1 mM neomycin, fixed, and stained with the nuclear stain bisbenzimide ('Hoechst staining'). The nuclei of cells in control utricles appeared oval and homogeneously stained with moderate intensity, whereas the nuclei of cells in neomycin-treated utricles had morphologies consistent with apoptosis. Those nuclei were intensely stained, branched, and irregularly shaped, indicative of massive chromatin structural changes. Greater numbers of extruded cells and condensed nuclei were observed in neomycin treated cultures as opposed to control cultures. A complete account of these results has appeared in published form (Matsui et al., 2002).

These findings demonstrated that vestibular hair cells undergo programmed cell death (PCD) after treatment with ototoxic antibiotics. We then focused on the identification of the signaling pathways that lead to the death of sensory cells in the vestibular organs. In many cell types, the PCD process initiates a cascade of intracellular events that culminates in the release of cytochrome c from mitochondria into the cytosol. This event results in the activation of caspases, leading to the degradation of the cell's nuclear proteins. In order to characterize the apoptotic pathways in hair cells, we have examined the effects of neomycin treatment and caspase inhibition on cytochrome c release in hair cells. Cultured chick utricles were treated with1 mM neomycin or control media for 6, 12, or 24 hours. Some cultured also received the general caspase inhibitor Boc-Asp-Fluoromethyl Ketone (BAF, 50 μM). Specimens were fixed and immunoreacted to detect cytosolic cytochrome c and co-labeled with Hoechst 33258, a nucleic acid stain. Labeled cells were counted in 8 randomly selected regions of each utricle. Few cells had cytosolic cytochrome c at 6 hours after addition of neomycin (7.2 ± 0.7), but that number increased between 12 and 24 hours (18.3 ± 1.8 and 27.0 ± 1.9). The number of pyknotic nuclei did not change between 6 and 12 hours, but increased about five-fold after 24 hours of neomycin treatment. In neomycin/BAF treated cultures, few cytochrome c^+ cells were observed at 6 hours, but this number increased by ~4x between 12 and 24 hours. In contrast, there was little change in the number of pyknotic nuclei between 6-24 hours. Also, few cytochrome c^+ cells and pyknotic cells were observed in control explants at any time point. These data imply that vestibular hair cells release cytochrome c from mitochondria in response to neomycin treatment, and that this release is not affected by caspase inhibition. Complete results are described in Matsui, Gale, and Warchol (2004).

Results of the previous experiments suggested that the activation of caspases must occur *after* the release of cytopchome c from hair cell mitochondria. An additional series of experiments was conducted in order to determine whether the release of cytochrome c is followed by the activation of effector caspases such as caspase-3. Cultured chick utricles were treated with 50 μM BAF (a general caspase inhibitor) and/or 1 mM neomycin, or control media for 1, 6, 12, 18, or 24 hours. Specimens were fixed and immunoreacted to detect activated caspase-3 and co-labeled with Hoechst 33258. Labeled cells were counted in 8 randomly selected regions of each utricle and the number of activated caspase-3^+ cells or pyknotic nuclei/25,000 μm^2 of sensory epithelium was calculated for each explant. Few cells contained activated capase-3 at 1-6 hours after neomycin exposure (4.2 ± 0.5 and 2.7 ± 0.4), but that number increased after 12 and 18 hours (9.2 ± 1.6 and 20.7 ± 0.8, respectively). The number of pyknotic nuclei was constant after 1-12 hours of neomycin treatment and then increased at 18 hours. In contrast, treatment with BAF resulted in few cells with activated caspase-3 or pyknotic nuclei. Similar findings were observed in control cultures. Taken together, these data

suggest that aminoglycoside-induced cell death is caspase-dependent and involves the activation of caspase-3. Complete results are described in Matsui, Gale, and Warchol (2004).

Role of Jun Kinase (JNK) in Sensory Cell Death

The c-Jun-N-terminal kinases (JNK's) have been implicated in many cellular processes including neuronal cell death and cell proliferation. Activation of JNK and phosphorylation of the transcription factor c-Jun occurs in mammalian hair cells following aminoglycoside-treatment (e.g., Pirvola et al., J Neurosci, 20:43). We examined the effects of CEP-11004, an indirect inhibitor of JNK activation, on the phosphorylation of c-Jun in hair cells and on hair cell death. Cultured chick utricles were treated with varying concentrations of CEP-11004 and 1 mM neomycin for 4-24 hours. They were then fixed, and immunoreacted with an antibody against calretinin, in order to label surviving hair cells. Hair cell densities were determined in both the extrastriolar and striolar regions. Additional specimens were immunoreacted to detect phosphorylated c-Jun or activated caspase 3, and co-labeled with Hoechst 33258. Labeled cells were counted and the number of phospho-c-Jun$^+$ cells, activated caspase-3$^+$ cells, or pyknotic nuclei/25,000 μm^2 of sensory epithelium was calculated for each explant. Neomycin-treatment alone significantly reduced hair cell densities (48% and 21% of controls respectively), but treatment with CEP-11004 promoted hair cell survival in the presence of neomycin in both the extrastriolar and striolar regions. Maximum protection was observed at 1600 nM (95% and 79% of control values). CEP-11004 also reduced the number of hair cells that were immunoreactive for phosphorylated c-Jun and activated caspase 3. These data imply that CEP-11004 promotes vestibular hair cell survival *in vitro*. Also, data on the timing of cellular changes after the onset of neomycin treatment indicated that the phosphorylation of c-Jun and JNK occurs upstream from the activation of caspases.

Evidence for Cell Cycle Entry During Hair Cell Death

Other experiments have focused on the identification of early events in the death of vestibular hair cells. Some neural cell types transiently enter the cell cycle before undergoing programmed cell death. Significantly, blocking S-phase entry in such cells can prevent PCD. We examined the effects of early cell cycle arrest on the survival of vestibular hair cells following treatment with neomycin. Cultured chick utricles were treated for 24 hours with 1 mM neomycin and 20 μM or 100 μM olomoucine, a cyclin dependent kinase (cdk) inhibitor that arrests cells at the G1/S and G2/M transitions of the cell cycle. Olomoucine treatment promoted hair cell survival in both the striolar and extrastriolar regions when compared to control cultures. In contrast, previous studies have demonstrated that cell cycle inhibitors that act beyond the G1/S interface (i.e., in S-phase) do not promote neuronal survival. Consistent with those results, treatment with aphidicolin, which prevents DNA synthesis after entry into S-phase, failed to prevent hair cell death in neomycin-treated cultures. Aphidicolin did, however, reduce supporting cell proliferation to 7% of control values. These results indicate that blocking transient entry into the cell cycle can increase hair cell survival after aminoglycoside treatment, but prevention of DNA replication after S-phase entry is not sufficient to promote hair cell survival.

Spontaneous Hair Cell Death and Supporting Cell Proliferation

Hair cells in the avian vestibular organs exhibit a unique pattern of turnover and replacement. The lifespan of vestibular hair cells in the avian ear is relatively brief (2-3 month). Those cells then die spontaneously and are replaced by new hair cells, which are produced by the division of epithelial supporting cells. We have examined the relationship between ongoing cell death and cell proliferation in the chick utricle. Utricles were incubated for 48 hours in media supplemented with 50 μM BAF or 0.1%

DMSO (controls). Following fixation, TUNEL$^+$ cells (a cellular label for apoptosis) were quantified in 25,000 μm^2 regions throughout the organ. BAF treatment reduced TUNEL-labeled cells to ~50% of control levels (7.0 ± 0.9 vs. 15.3 ± 1.4). Further experiments examined the effects of reducing ongoing HC death on the rate of supporting cell proliferation. Utricles were cultured as described above, but BrdU was added to the culture media during the last 4 hours *in vitro*. Proliferating cells were quantified in 25,000 μm^2 regions throughout the organ. BAF treatment reduced BrdU$^+$ cells to ~50% of control levels (22.7 ± 2.0 vs. 41.9 ± 3.1). In order to determine whether caspase inhibitors can directly affect supporting cell proliferation, isolated sheets of utricular sensory epithelia were cultured in media supplemented with 50 μM BAF or 0.1% DMSO. BrdU was added to the medium for the final 4 hours *in vitro*. Similar levels of cell proliferation were observed in both the BAF-treated and control cultures. The results suggest that ongoing HC death stimulates supporting cell proliferation in the mature utricle. These data have appeared in published form (Matsui et al., 2002)

Inhibition of Caspases Prevents Vestibular Ototoxicity *In Vivo*

The studies described above demonstrate that hair cell survival after *in vitro* treatment with ototoxic agents can be enhanced by co-application of caspase inhibitors. Based on that result, we examined the effects of caspase inhibition on hair cell death *in vivo*. Our studies used systemic injections of aminoglycosides and infusion of caspase inhibitors into the inner ear. In initial studies, chickens were implanted with osmotic pumps containing 100 μM zVAD (general caspase inhibitor) or carrier. The catheter attached to the pump was implanted into the vestibule (Roberson et al., Hear. Res. 141:155). One day following the surgery, the animals received daily IM injections of either streptomycin (1200 mg/kg) or saline for 5 consecutive days. Utricles were then removed, fixed and hair cells were identified by immunoreactivity to calretinin. Hair cells were quantified in 10,000 μm^2 regions of each specimen. Direct infusion of zVAD into the vestibule increased hair cell survival in both the striolar and extrastriolar regions of streptomycin-treated utricles. Following treatment with 100 μM zVAD and streptomycin, sampled regions contained 55 ± 2 hair cells/10,000 μm^2 in the striolar region and 144 ± 13 hair cells/10,000 μm^2 in the extrastriolar region. Treatment with 100 μM zVAD and streptomycin resulted in 69 ± 4 hair cells/10,000 μm^2 in the striolar region and 176 ± 4 hair cells/10,000 μm^2 in the extrastriolar region. Following treatment with carrier and streptomycin, sampled areas contained 28 ± 6 hair cells in the striolar region and 76 ± 8 hair cells in the extrastriolar region. Similar densities were obtained with animals receiving streptomycin treatment alone. Animals that received saline alone had 75 ± 3 hair cells/10,000 μm^2 in the striolar region and 153 ± 3 hair cells/10,000 μm^2 in extrastriolar region. These are the fist results to indicate that caspase inhibitors promote the survival of vestibular hair cells *in vivo* following treatment with ototoxic antibiotics.

A second series of experiments demonstrated that systemic application of zVAD can also prevent hair cell death following streptomycin treatment. We then asked whether these rescued hair cells were still able to function as sensory receptors. Animals received a five day regimen of streptomycin and zVAD (experimental group) or streptomycin alone (control group). Vestibular function was assessed by quantifying the vestibulo-ocular reflex (VOR). The VOR gains obtained from animals that received streptomycin and zVAD were significantly higher than those obtained from animals that received streptomycin alone. Consistent with this result, the vestibular organs of the zVAD-treated animals contained many more hair cells than did the streptomycin animals. These results suggest that blocking hair cell death by preventing caspase activation (which occurs quite late in the cell death pathway) results in preservation of sensory function. A complete account of these results has been published (Matsui et al., 2003).

Publications Supported by NAG2-1364

J.I. Matsui, J.E. Gale, and **M.E. Warchol** (to be submited, fall 2003) Mitochondrial and Caspase Regulation of Aminoglycoside-Induced Hair Cell Death. Journal of Neurobiology.

L.L. Cunningham, J.I. Matsui, **M.E. Warchol** and E.W. Rubel (submitted) Overexpression of *Bcl-2* Prevents Aminoglycoside-Induced Hair Cell Death and Caspase-9 Activation in the Adult Mouse Utricle in vivo. *Journal of Neurobiology*

J.I. Matsui, A. Haque, D. Huss, E.P. Messana, J.A. Alosi, D.W. Roberson, D.A. Cotanche., J.D. Dickman, and **M.E. Warchol** (2003) Caspase Inhibitors Promote Vestibular Hair Cell Function and Survival Following Aminoglycoside Treatment *in vivo*. *Journal of Neuroscience* 23: 6111-6122

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Abstracts and Meeting Presentations Supported by NAG2-1364

J.I. Matsui and M.E. Warchol (2003) JNK Signaling in Hair Cell Death and Regeneration. Abstract 79. 26th Midwinter Meeting of the Association for Research in Otolaryngology. Daytona Beach FL

J.I. Matsui, E.P. Messana, J.A. Alosi, D.W. Roberson, D.A. Cotanche, and M.E. Warchol (2002) Hair Cell Survival Following Aminoglycoside Treatment with Caspase Inhibitors *in vivo*. 25th Midwinter Meeting of the Association for Research in Otolaryngology. St. Petersburg FL

J.I. Matsui and M.E. Warchol (2001) Activated Caspase-3 Expression in Chick Vestibular Hair Cells Following Neomycin Treatment. Molecular Biology of Hearing and Deafness, Bethesda, MD

J.E. Gale and M.E. Warchol (2001) Rapid Intercellular Calcium Signaling and Actin Cable Formation at Wound Sites After Laser Lesioning in Chick Vestibular Epithelial Cultures. 24th Midwinter Meeting of the Association for Research in Otolaryngology. St. Petersburg FL

J.I. Matsui and M.E. Warchol (2001) Cytochrome c Redistribution in Hair Cells following Neomycin Treatment. 24th Midwinter Meeting of the Association for Research in Otolaryngology.
St. Petersburg FL

M.E. Warchol, J.I. Matsui, J.M. Olgilvie, and E.L. Simkus (2000) Ototoxicity and Immune Regulation of Hair Cell Regeneration. NYAS Symposium on Vestibular Labyrinth in Health and Disease. St. Louis MO

J.I. Matsui, J.M. Olgilvie, and M.E. Warchol (2000) A Causative Relationship Between Ongoing Hair Cell Death and Supporting Cell Proliferation in Avian Vestibular Organs. Society for Neuroscience Annual Meeting

J. I. Matsui and M.E. Warchol (2000) Early Cell Cycle Arrest Promotes Hair Cell Survival After Aminoglycoside Treatment. 23rd Midwinter Meeting of the Association for Research in Otolaryngology. St. Petersburg FL